

Simvastatin Reduces Neointimal Thickening in Low-Density Lipoprotein Receptor-Deficient Mice After Experimental Angioplasty Without Changing Plasma Lipids

Zhiping Chen, MS; Tatsuya Fukutomi, MD; Alexandre C. Zago, MD; Raila Ehlers, MD; Patricia A. Detmers, PhD; Samuel D. Wright, PhD; Campbell Rogers, MD; Daniel I. Simon, MD

Background—Statins exert antiinflammatory and antiproliferative actions independent of cholesterol lowering. To determine whether these actions might affect neointimal formation, we investigated the effect of simvastatin on the response to experimental angioplasty in LDL receptor-deficient ($LDLR^{-/-}$) mice, a model of hypercholesterolemia in which changes in plasma lipids are not observed in response to simvastatin.

Methods and Results—Carotid artery dilation (2.5 atm) and complete endothelial denudation were performed in male C57BL/6J $LDLR^{-/-}$ mice treated with low-dose (2 mg/kg) or high-dose (20 mg/kg) simvastatin or vehicle subcutaneously 72 hours before and then daily after injury. After 7 and 28 days, intimal and medial sizes were measured and the intima to media area ratio (I:M) was calculated. Total plasma cholesterol and triglyceride levels were similar in simvastatin- and vehicle-treated mice. Intimal thickening and I:M were reduced significantly by low- and high-dose simvastatin compared with vehicle alone. Simvastatin treatment was associated with reduced cellular proliferation (BrdU), leukocyte accumulation (CD45), and platelet-derived growth factor-induced phosphorylation of the survival factor Akt and increased apoptosis after injury.

Conclusions—Simvastatin modulates vascular repair after injury in the absence of lipid-lowering effects. Although the mechanisms are not yet established, additional research may lead to new understanding of the actions of statins and novel therapeutic interventions for preventing restenosis. (*Circulation*. 2002;106:20-23.)

Key Words: restenosis ■ statins ■ inflammation ■ apoptosis

Statin drugs inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the first committed step of sterol synthesis, and lower plasma cholesterol levels. In large clinical trials, statins have been shown to reduce coronary events in primary or secondary prevention settings.¹ Effects on clinical and angiographic restenosis after coronary intervention, however, have not been conclusively demonstrated. Several clinical studies have failed to demonstrate a link between statin therapy and the risk of restenosis after balloon angioplasty,² whereas more recent studies suggest that statins may reduce restenosis after stenting.³

Statins are known to have broad effects in addition to lowering plasma cholesterol. The product of HMG-CoA reductase, mevalonate, is an important precursor for many isoprenoids, thereby endowing statins with the ability to directly alter cellular events other than cholesterol synthesis. For example, the isoprenoids farnesylpyrophosphate and geranylgeranylpyrophosphate play important roles in signal transduction in cellular migration, proliferation, and survival via their attachment to critical signaling proteins, such as Ras and Rho.⁴

We used a hyperlipidemic model, the $LDLR^{-/-}$ mouse, to test the antiinflammatory and antiproliferative actions of simvastatin on neointimal thickening after experimental angioplasty in an atherosclerotic background. An essential feature of the chosen model is that simvastatin does not affect plasma lipid levels in mice, allowing the study of effects of simvastatin distinct from cholesterol lowering.

Methods

Carotid Injury

Male $LDLR^{-/-}$ C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me), maintained on a high-fat (20.1%) diet containing 1.25% cholesterol for 12 weeks after weaning, underwent unilateral carotid artery dilation (2.5 atm) and complete endothelial denudation.⁵ Animal care and procedures were reviewed and approved by Harvard Medical School Standing Committee on Animals and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Simvastatin Treatment

Treatments were via subcutaneous injection 72 hours before and daily after injury. $LDLR^{-/-}$ mice were divided into 3 treatment

Received March 29, 2002; revision received May 8, 2002; accepted May 9, 2002.

From the Cardiovascular Division (Z.C., T.F., A.C.Z., R.E., C.R., D.I.S.), Brigham and Women's Hospital, Boston, Mass; Harvard-MIT Division of Health Sciences and Technology (C.R.), Cambridge, Mass; and Merck Research Laboratories (P.A.D., S.D.W.), Rahway, NJ.

Correspondence to Daniel I. Simon, MD, Cardiovascular Division, Brigham and Women's Hospital, 75 Francis St, Tower 3, Boston, MA 02115. E-mail: dsimon@rics.bwh.harvard.edu

© 2002 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

DOI: 10.1161/01.CIR.0000022843.76104.01

**Quantitative Morphometry and Immunohistochemical Analysis of Mouse Carotid Arteries
After Injury**

	Simvastatin			ANOVA	P	
	Vehicle	Low	High		Vehicle vs Low	Vehicle vs High
Cholesterol, 21 d, mg/dL	856±89	922±167	1130±279	0.074	NS	NS
Triglyceride, 21 d, mg/dL	216±36	234±43	223±44	0.751	NS	NS
Intimal area, mm ²						
7 d	0.010±0.004	0.006±0.004	0.006±0.004	0.320	NS	NS
28 d	0.047±0.023	0.021±0.013	0.019±0.015	0.004	0.011	0.012
Medial area, mm ²						
28 d	0.078±0.015	0.063±0.014	0.074±0.031	0.299	NS	NS
EEL, mm ²						
7 d	0.104±0.013	0.113±0.035	0.103±0.014	0.778	NS	NS
28 d	0.223±0.032	0.191±0.040	0.192±0.053	0.184	NS	NS
BrdU+ cells, %						
Media, 7 d	19.2±2.8	12.0±5.8	11.0±4.5	0.299	NS	NS
Intima, 28 d	4.6±1.8	1.7±0.5	1.9±0.8	0.0184	0.042	0.050
CD45+ cells, %						
Intima, 7 d	59.2±11.9	44.2±5.2	39.1±12.8	0.0429	0.047	0.049
Intima, 28 d	34.4±3.6	24.3±2.2	20.9±8.0	0.0120	0.026	0.025
TUNEL+ cells, %						
Intima, 7 d	3.5±1.1	6.9±3.3	9.2±4.2	0.0872	0.193	0.039
Media, 7 d	2.5±0.4	4.2±0.3	5.8±1.0	0.0002	0.002	0.001

groups: PBS vehicle (control group) or 2 mg/kg (low-dose) or 20 mg/kg (high-dose) alkaline-hydrolyzed simvastatin.⁶

Lipid Analysis

Blood was collected via retro-orbital puncture into heparin-coated capillary tubes. Plasma cholesterol and triglyceride measurements were performed as reported.⁷

Tissue Harvesting and Analysis

Carotid arteries were harvested and processed for quantitative morphometry 7 days (control, n=5; low-dose, n=5; high-dose, n=4) or 28 days (control, n=9; low-dose, n=10; high-dose, n=7) after vascular injury.⁵ Standard avidin-biotin procedures for mouse leukocytes (CD45) and macrophages (Mac-3) (PharMingen, San Diego, Calif), BrdU (DAKO, Carpinteria, Calif), and smooth muscle cell (SMC) α -actin (DAKO) were used for immunohistochemistry. Apoptotic cells were detected by the TUNEL method using Apo Tag (Intergen). Immunostained sections were quantified as the number of immunostained-positive cells per total number of nuclei.

Ex Vivo Akt Signaling Assay

Aortas were harvested from all animals, opened longitudinally, and incubated with 30 ng/mL platelet-derived growth factor (PDGF)-BB (R&D Systems, Minneapolis, Minn) for 15 minutes at 37°C. Aortic lysates were prepared⁸ and then subjected to Western analysis using antibodies to Akt and Phospho-Akt (Ser473) (Cell Signaling Technology, Beverly, Mass).

Data Analysis

All data are presented as mean \pm SD. Statistical comparisons of the principal end points were performed using one-way ANOVA to determine a difference in mean values between the 3 groups, followed by *t* tests for the 3 pair-wise comparisons when the ANOVA false-positive rate was <5%. For ANOVA with a false-

positive rate of >5%, the pair-wise comparisons were reported to be statistically nonsignificant (NS). For the primary study end point of intimal area 28 days after injury, a Bonferroni corrective for 3 pair-wise comparisons was applied, in which the *t* test *P*<0.0167 was used to signify a false-positive rate of 5%.

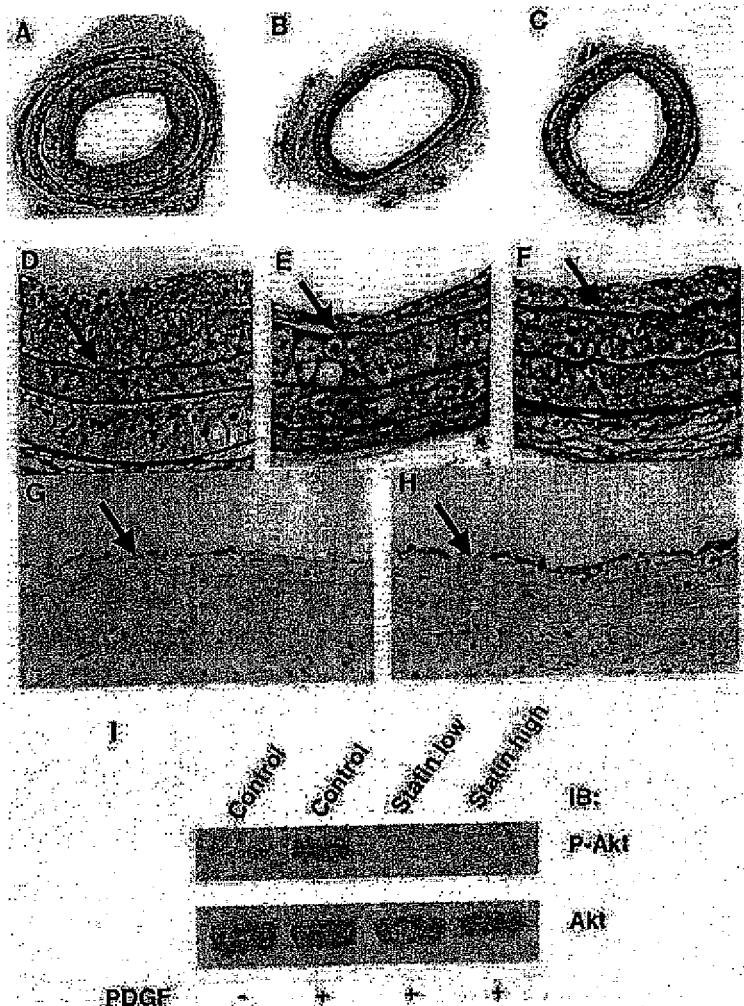
Results

Simvastatin Does Not Alter Plasma Lipids in *LDLR^{-/-}* Mice

We sought evidence that simvastatin modulates vascular repair independent of cholesterol lowering. To determine whether plasma cholesterol is unresponsive to simvastatin in *LDLR^{-/-}* mice, as it is in normal⁹ and apoE-deficient⁷ mice, we dosed *LDLR^{-/-}* animals with 2 mg/kg simvastatin, 20 mg/kg simvastatin, or vehicle control and measured plasma lipid levels. Simvastatin did not alter plasma cholesterol or triglyceride levels in *LDLR^{-/-}* mice at either of the 2 doses tested (Table).

Simvastatin Decreases Neointimal Thickening, Cellular Proliferation, and Leukocyte Accumulation After Carotid Injury

Carotid artery dilation and complete endothelial denudation were performed in *LDLR^{-/-}* mice treated with 2 or 20 mg/kg simvastatin or vehicle subcutaneously 72 hours before and then daily after injury until euthanasia. In mice receiving vehicle, intimal thickening began by 7 days after injury and progressed significantly between 7 days (0.010 \pm 0.004 mm²) and 28 days (0.047 \pm 0.023 mm²). Low- and high-dose simvastatin reduced intimal thickening at 28 days by 55%



Photomicrographs of mouse carotid arteries after injury (A through H). Ver-Hoefff elastin stain 28 days after injury: vehicle (A); low-dose simvastatin (B); high-dose simvastatin (original magnification $\times 38$) (C); vehicle (D); low-dose simvastatin (E); and high-dose simvastatin ($\times 150$) (F). Neointima separates the internal elastic lamina (arrows) from the lumen. Apoptotic (TUNEL-positive) cells 7 days after injury: vehicle (G); high-dose simvastatin ($\times 150$) (H). Simvastatin and Akt signalling (I). Aortae were harvested from mice treated with simvastatin or vehicle and incubated with PDGF-BB. Aortic lysates were immunoblotted sequentially using antibodies to Akt and Phospho-Akt (Ser473).

($P=0.012$) and 60% ($P=0.011$), respectively (Figure, panels A through F, Table). Medial area was unaffected by simvastatin treatment. I:M at 28 days in control mice was 0.64 ± 0.37 and was reduced 50% by low-dose ($P=0.036$) and 62% by high-dose ($P=0.012$) simvastatin. Intimal and medial thickening were accompanied by progressive vessel enlargement (ie, positive remodeling), as determined by external elastic lamina area measurements over time, which was comparable in vehicle- and simvastatin-treated mice.

We assessed cellular proliferation by quantifying incorporation of BrdU. Substantial proliferation was observed 7 days after injury in control vessels (19.2% of medial cells), and proliferation was still evident at 28 days (4.6% of intimal cells). Low- and high-dose simvastatin reduced medial proliferation at 7 days by 38% and 43%, respectively, and intimal proliferation at 28 days by 63% ($P=0.042$) and 59% ($P=0.050$) (Table).

Immunohistochemistry was performed to identify the cellular components of the neointima 28 days after injury. In vehicle-treated animals, 48% of cells were SMCs (α -actin-positive) and 34% were monocytes or macrophages (CD45- and Mac3-positive). Altered leukocyte accumulation within vessels was observed in simvastatin-treated mice. Inflammatory cells (CD45-positive) invading the intima were reduced

by 25% to 34% ($P<0.05$) at 7 days and 29% to 39% ($P<0.03$) at 28 days in simvastatin-treated compared with control mice.

Simvastatin Increases Apoptosis

Because statins prevent isoprenylation of Rho proteins and their translocation to the membrane fraction, and because there is increasing evidence that Rho activates signals that regulate apoptosis,¹⁰ we investigated the effects of simvastatin on apoptosis after injury. Low- and high-dose simvastatin significantly increased the number of apoptotic (TUNEL-positive) cells in the intima (by 197% and 263%, respectively) and media (168% and 232%, respectively) at 7 days compared with control (Table, Figure, panels G and H).

To identify a biochemical correlate of simvastatin action promoting apoptosis, we examined signaling of the survival factor, Akt, in arteries from mice treated with simvastatin. Injured carotid arteries are completely devoid of endothelium and lined with a platelet monolayer.⁵ Therefore, we examined PDGF-induced phosphorylation and activation of Akt by Western blot analysis of aortic samples from mice treated with low- and high-dose simvastatin or vehicle for 7 days. PDGF-induced phosphorylation of Akt was impaired in the aortae of simvastatin-treated mice (Figure, panel I).

Discussion

Our study provides definitive *in vivo* evidence that simvastatin inhibits neointimal thickening in a cholesterol-independent manner accompanied by reduced vascular inflammation and proliferation and increased apoptosis. These results establish a role for statins in inhibiting neointimal formation after experimental angioplasty in a setting in which simvastatin did not alter plasma lipids.

Restenosis is a complex cascade of wound-healing responses to vascular injury, characterized by thrombosis, inflammation, cellular proliferation/migration, and extracellular matrix deposition. Increasing evidence suggests that antiinflammatory⁷ and antiproliferative¹¹ effects of statins play important roles in attenuating atherosclerosis,^{1,7} transplant vasculopathy,¹² and restenosis.³ Statins inhibit the synthesis of isoprenoid intermediates that are important lipid attachments for signaling proteins, including Ras and the Rho family of small GTP-binding proteins (eg, Rho, Rac, and Cdc42).⁴ Rho is implicated in various biological functions relevant to vascular injury, including cellular migration, proliferation, and survival.^{10,13} Statins attenuate vascular SMC proliferation *in vitro* by decreasing Rho geranylgeranylation and membrane localization and inhibiting Cdk activity.¹¹

We provide biochemical evidence that PDGF-induced phosphorylation of Akt is inhibited in aortic tissue from simvastatin-treated mice. Akt functions as an antiapoptotic protein, protecting against cell death induced by growth factor withdrawal or ischemia-reperfusion injury.¹⁴ The effects of statins on Akt signaling seem to be tissue-specific. Statins rapidly activate Akt signaling in endothelial cells, enhance phosphorylation of endothelial NO synthase, and inhibit apoptosis.¹⁵ In contrast, statins impair Akt activation in SMCs,¹⁶ leading to diminished SMC proliferation and induction of apoptosis via effects on phosphatidylinositol-3 kinase or Rho.¹¹ These divergent actions of statins on Akt activation in endothelial cells and SMCs may act in synchrony to diminish neointimal thickening after denuding injury.

Prior clinical trials of statins after balloon angioplasty have failed to show a reduction in restenosis,² likely because of the predominant role of vascular remodeling rather than neointimal thickening in this setting.¹⁷ However, recent studies of statin use after stenting, with minimal remodeling and profound neointimal thickening,¹⁷ have suggested benefit.³

Our results support the hypothesis that simvastatin has antiinflammatory, antiproliferative, and proapoptotic actions relevant to preventing restenosis. Although mechanisms are not yet established, additional research may lead to new

understanding of the actions of statins, additional impetus for broad statin use after vascular intervention independent of lipid profile, and novel therapies for preventing restenosis.

Acknowledgments

This research was supported by National Institutes of Health Grants R01 HL57506 (to Dr Simon), R01 DK55656 (to Dr Simon), and K08 HL03104 (to Dr Rogers) and a Merck Medical School Grant (to Dr Simon).

References

- Bucher HC, Griffith LE, Guyatt GH. Systematic review on the risk and benefit of different cholesterol-lowering interventions. *Arterioscler Thromb Vasc Biol.* 1999;19:187-195.
- Weintraub WS, Bocuzzi SJ, Klein JL, et al. Lack of effect of lovastatin on restenosis after coronary angioplasty: Lovastatin Restenosis Trial Study Group. *N Engl J Med.* 1994;331:1331-1337.
- Walter DH, Schachinger V, Elsner M, et al. Effect of statin therapy on restenosis after coronary stent implantation. *Am J Cardiol.* 2000;85:962-968.
- Casey PJ. Protein lipidation in cell signaling. *Science.* 1995;268:221-225.
- Simon DI, Chen Z, Seifert P, et al. Decreased neointimal formation in Mac-1(-) mice reveals a role for inflammation in vascular repair after angioplasty. *J Clin Invest.* 2000;105:293-300.
- Endres M, Laufs U, Huang Z, et al. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci USA.* 1998;95:8880-8885.
- Sparrow CP, Burton CA, Hernandez M, et al. Simvastatin has antiinflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol.* 2001;21:115-121.
- DiChiara MR, Kiely JM, Gimbrone MA Jr, et al. Inhibition of B-selectin gene expression by transforming growth factor β in endothelial cells involves coactivator integration of Smad and nuclear factor κ B-mediated signals. *J Exp Med.* 2000;192:695-704.
- Endo A, Tsujita Y, Kuroda M, et al. Effects of ML-236B on cholesterol metabolism in mice and rats: lack of hypocholesterolemic activity in normal animals. *Biochim Biophys Acta.* 1979;575:266-276.
- van Nieuw Amerongen GR, van Hinsbergh VW. Cytoskeletal effects of rho-like small guanine nucleotide-binding proteins in the vascular system. *Arterioscler Thromb Vasc Biol.* 2001;21:300-311.
- Laufs U, Marra D, Node K, et al. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate vascular smooth muscle cell proliferation by preventing rho GTPase-induced down-regulation of p27Kip1. *J Biol Chem.* 1999;274:21926-21931.
- Kobashigawa JA, Katzenbach S, Laks H, et al. Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med.* 1995;333:621-627.
- Shibata R, Kai H, Seki Y, et al. Role of Rho-Associated kinase in neointima formation after vascular injury. *Circulation.* 2001;103:284-289.
- Fujio Y, Nguyen T, Wencker D, et al. Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in mouse heart. *Circulation.* 2000;101:660-667.
- Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med.* 2000;6:1004-1010.
- Weiss RH, Ramirez A, Joo A. Short-term pravastatin mediates growth inhibition and apoptosis, independently of Ras, via the signaling proteins p27Kip1 and PI3 kinase. *J Am Soc Nephrol.* 1999;10:1880-1890.
- Hoffmann R, Mintz GS, Dussaillant GR, et al. Patterns and mechanisms of in-stent restenosis: a serial intravascular ultrasound study. *Circulation.* 1996;94:1247-1254.